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Antisense Cyclin D1 Induces Apoptosis and Tumor Shrinkage in Human Squamous Carcinomas[1]

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ABSTRACT

Cyclin D1 plays an essential regulatory role in the G_1 phase of the cell cycle. The cyclin D1 gene is amplified in 20–50% of squamous cell carcinomas (SCCs), and the protein is overexpressed in up to 80% of SCCs. Our hypothesis was that gene transduction of antisense (AS) cyclin D1 in human SCCs *in vivo* would result in tumor reduction. A cyclin D1 cDNA was inserted into an E1/E3-deficient serotype 5 adenovirus (AS cyclin D1) in an AS orientation using homologous recombination. AS cyclin D1 transduction suppressed cyclin D1 protein expression in both cultured cells and tumors. AS cyclin D1 significantly inhibited cell proliferation by both [^{3}H]thymidine incorporation in six SCC cell lines ($P = 0.01–0.001$) and the conversion of tetrazolium salt to formazan in four SCC cell lines ($P = 0.01–0.004$). Apoptosis detected in >25% of cells in each cell line 48 h after AS cyclin D1 transduction paralleled the reduction in cyclin D1 protein. Preformed SCCs transduced with AS cyclin D1 were significantly inhibited ($P = 0.002–0.005$), and apoptosis was prominent in the AS cyclin D1-treated tumors, but not in tumors treated with the control vector. These data extend prior *in vitro* and *ex vivo* results and indicate that AS cyclin D1 suppresses SCC growth both *in vitro* and *in vivo* through suppression of cyclin D1 protein expression, leading to cellular apoptosis. Our findings suggest that cyclin D1 may have a role in cell survival and that cyclin D1 AS therapy may be useful as an adjunct to standard treatment for SCC.

INTRODUCTION

There is substantial evidence that critical regulatory steps occur during G_1 of the cell cycle that determine whether or not a cell will synthesize new DNA and divide. The regulators of G_1, particularly late G_1, are also frequent targets for mutations (1). The major regulator appears to be pRb,[3] the protein product of the retinoblastoma gene (2). When hypophosphorylated, pRb inhibits cell growth by binding to and preventing the function of transcription factors, including members of the E2F family (1). Phosphorylation of pRb in mid to late G_1 releases the transcription factor(s) bound by pRb, which leads to DNA synthesis (3).

D-type cyclins bind to the pocket domain of hypophosphorylated pRb through their L*X*C*X*E sequences, which they share with several DNA tumor viruses (4). Cyclin D1 is a proto-oncogenic regulator of the G_1-S-phase checkpoint in the cell cycle that has been implicated in the pathogenesis of several types of cancer, including SCC, in which the cyclin D1 gene is amplified, and the gene product is overexpressed (5). Cyclin D1 appears to function upstream of pRb by binding to cdk4 or cdk6, leading to pRb phosphorylation (6). The fact that cells with nonfunctional pRb (7) do not require cyclin D1 to divide suggests that cyclin D1 operates through pRb (8).

Cytogenetic analyses of head and neck SCC have demonstrated a breakpoint on chromosome 11q13 (9). Amplification of the oncogenes *int-2, hst-1*, and *prad1* (bcl-1, CCND1, and cyclin D1) in the 11q13 amplicon have been reported previously (10). Of the three genes, only *prad1* is expressed. Amplification of the cyclin D1 gene is present in 20–50% of SCCs, and the protein is overexpressed in up to 80% of SCCs (11, 12). Overexpression of cyclin D1 in cultured cells leads to a more rapid transversion through the G_1 phase of the cell cycle and entry into S phase (13, 14). Cyclin D1 can cooperate with *ras* (15) and can complement a defective *Ela* adenoviral gene (16) to function as an oncogene. In a transgenic mouse model (17), overexpression of human cyclin D1 led to dysplasic progression in the oral cavity and esophagus, suggesting that cyclin D1 overexpression can transform squamous epithelium.

Other gene products that help regulate the G_1 progression through pRb include p16/cdkn2, cdk4, and cdk6 (18). Whereas deletions and mutations in p16 in primary SCCs are uncommon, occurring in 0–20% of cases (19–21), methylation appears to be a major mechanism of p16 gene inactivation in SCCs (22), occurring in up to 43% of cases. Methylation of CpG islands of growth-regulatory genes leading to transcriptional activation is a common feature in head and neck, lung, and neuroblastoma carcinogenesis (22). cdk4 and cdk6 gene mutations are rare in cancer of any origin (23). We therefore elected to study cyclin D1, which is frequently mutated and overexpressed in SCCs, suggesting that alteration of the gene is an important event in SCC development and/or progression.

To gain a better understanding of the importance of cyclin D1 in squamous cell carcinogenesis, we inserted the entire open reading frame of cyclin D1 in AS orientation into an Ad (AS cyclin D1) and evaluated the effect of cyclin D1 inhibition on protein expression, cell growth, and apoptosis, both *in vitro* and, more importantly, *in vivo*. A CMV-driven adenoviral vector was chosen for maximum intracellular expression of the transgene. Our findings demonstrate that AS cyclin D1 leads to protein inhibition, decreased cell growth, and apoptosis in all SCC cell lines tested that have functional pRb. We report, to our knowledge, the first clear evidence that AS cyclin D1 is effective *in vivo* in tumors of common lineage and that tumor shrinkage is associated with a dramatic increase in apoptosis, demonstrating the potential efficacy of AS cyclin D1 gene therapy to treat SCCs.

MATERIALS AND METHODS

Cell Culture and Proliferation Assays. Six human SCC cell lines [three from the head and neck (SCC 9, A253, and Det562), one from facial skin (SCC 13), one from vulvar skin (A431), and one from the cervix (C33A)] were kindly provided by Dr. James Rheinwald (SCC 9 and SCC 13; Brigham and Women's Hospital, Boston, MA) or obtained from the American Type Culture Collection (A253, Det562, A431, and C33A; Manassas, VA). The SCC cell lines, which were previously maintained in DMEM supplemented with 10% FCS, were weaned off serum and grown at a 4:1 ratio in MCDB 201/L15 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 5 μg/ml insulin (SCC medium). Transcomplementing 293 cells (24) immortalized and transformed by adenovirus E1a and Elb were obtained from the Vector Core at the Institute for Human Gene Therapy (University of Pennsylvania, Philadelphia, PA) and grown in DMEM supplemented with 10% FCS. Unless otherwise noted, all tissue culture reagents were purchased from Sigma.

[^{3}H]Thymidine and MTT *in vitro* proliferation assays were performed. For the former, four to six replicates (2×10^4 cells/microtiter well) were mock-transduced or transduced (10, 50, and 100 pfu) with AS cyclin D1 or a control vector (an E1-deleted Ad5 without a gene insert or an E1/E3-deleted Ad5 containing the

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[3] The abbreviations used are: pRb, retinoblastoma protein; Ad, replication-deficient adenovirus; AS, antisense; cdk, cyclin-dependent kinase; CMV, cytomegalovirus; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide); pfu, plaque-forming unit; SCC, squamous cell carcinoma; SCID, severe combined immunodeficient; mAb, monoclonal antibody.

LacZ gene). Thirty h later, 1 μCi of [^{3}H]thymidine was added for 18 h, medium was removed, and the cells were detached with 0.06% trypsin, harvested with a Filtermate 196 Cell Harvester (Packard Instrument Co., Meriden, CT), and assessed for radioactivity (counts/3 min) using a Matrix 9600 Direct Beta Counter (Packard Instrument Co.). In MTT assays, 5×10^3 cells were added (five wells/treatment) in 96-well plates and mock-transduced or transduced with AS cyclin D1 or a control vector for 48 h. Dye and solubilization/stop solutions were added according to the manufacturer's instructions (Promega, Madison, WI), and absorbance at 570 nm was measured using an EL 312e Microplate Reader (Bio-Tek Instruments, Winooski, VT). Wilcoxon's rank-sum test was used to determine any significant difference in growth between the groups.

For *in vivo* studies, cell lines were grown to 70–80% confluence and detached with 0.06% trypsin, cells were counted, and 10^7 cells were injected s.c. into the dorsum of SCID mice just medial to the right hind limb. After tumors had grown to a minimum volume of 100 mm^3, a single injection containing 5×10^8 pfu AS cyclin D1 or control in 50 μl of SCC medium was administered into five to six replicate tumors using a tuberculin syringe. All animals were sacrificed 7 days after treatment. For statistical analysis, tumor volumes at baseline and at day 4 and day 7 after viral transduction were fitted to an exponential curve $y = A \exp(\beta t)$, where β is the tumor regression rate estimated for each mouse from the three observations. β values in each group were rank-ordered and analyzed by the Wilcoxon two-sample (one-sided) procedure to test the hypothesis of equal rates of tumor regression among groups *versus* the alternative that treated tumors regress faster.

Cell Cycle and Apoptosis Assays. Cells from each cell line were grown to 70–80% confluence, spent medium was removed, and fresh SCC medium containing adenoviral vectors was added. Twenty-four, 48, and 72 h after infection, cells were detached with 0.06% trypsin and fixed in 80% ethanol. After pelleting and ethanol removal, cells were resuspended in propidium iodide/RNase for a minimum of 20 min. Cell cycle analysis, including the sub-G_0 or apoptotic cell fraction, was then carried out using an EPICS XL flow cytometer (Coulter Corp., Hialeah, FL). Wilcoxon's rank-sum test was used to determine any significant difference in growth between the groups.

Apoptosis was also analyzed *in vivo*. Formalin-fixed slides containing A253 tumors formed in SCID mice were deparaffinized through changes of xylene and ethanol and rehydrated in water. Proteinase K (20 μg/ml) was applied to the slides for 8 min (Oncor, Gaithersburg, MD) and quenched with H_2O_2. The slides were rinsed in tap water, and terminal deoxynucleotidyltransferase enzyme was applied, followed by anti-digoxigenin-peroxidase and the chromagen 3′,3′-diaminobenzidine. Slides were counterstained with hematoxylin, and a coverslip was applied.

Construction of Replication-defective Adenoviral Vector. A 1.1-kb PCR product including the entire cyclin D1 open reading frame was inserted into the *Pst*I/*Eco*RI modified (Vector Core, Institute for Human Gene Therapy) multiple cloning site of pSL301 (Invitrogen, Carlsbad, CA). The resulting plasmid was cut with *Not*I and subcloned into the *Not*I site of the adenoviral vector pAd.CMV-Link.1 (25). pAd.CMV-Link1 was then cut with *Eco*RI to determine cyclin D1 orientation. After linearization with *Nhe*I, pAd.CMV-Link1 was cotransfected into 293 cells containing the E1 gene of Ad5 with *Cla*I-digested adenoviral DNA (dl7001) lacking the E1 and E3 regions (26) using calcium phosphate precipitation. The vectors were incubated in serum-free DMEM/10% FCS. After 5 h, a 10% glycerol shock was administered, and the cells were grown with an overlay of 0.8% bactoagar (Difco Laboratories, Detroit, MI) containing 2% FCS/12.5 mM $MgCl_2$ in MEM. Fresh overlay was added every 3–4 days until plaques appeared, typically by day 16.

Putative plaques were expanded and screened by restriction fragment and Southern blot analyses (27), a positive AS cyclin D1 plaque was propagated in 293 cells (28), the virus was released by freeze-thawing and purified by CsCl gradient centrifugation, and the final pfu was determined by titration under an agar overlay (29). An adenoviral vector with the reporter gene LacZ and an E1 and E3 gene-deleted adenovirus were obtained from the Vector Core of the Institute for Human Gene Therapy. Both vectors were used as controls.

Detection of Cyclin D1 by Western Blotting and Immunohistochemistry. One, 6, 24, and 48 h after gene transduction with adenoviral vectors, a SDS-based buffer was added to the cells, and total protein concentration was determined using the Pierce BCA Protein Assay Reagent Kit (Rockford, IL). Samples (100 μg) were separated electrophoretically under reducing conditions in a discontinuous 12% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. This procedure was performed twice. After transfer, membranes were blocked with 5% nonfat dry milk and probed with a mouse mAb to either β-actin (clone AC-15; Sigma) or cyclin D1 (clone HD-11; Santa Cruz Biotechnology, Santa Cruz, CA), followed by a phosphatase-conjugated goat antimouse IgG (Jackson ImmunoResearch, West Grove, PA) and the substrates 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium.

Formalin-fixed SCCs formed in SCID mice and treated with either LacZ or AS cyclin D1 were cut into 5-μm sections and placed on poly-L-lysine-coated glass slides. After boiling in distilled water for 10 min, slides were incubated with an anti-cyclin D1 mAb (Ab-3; Calbiochem, Cambridge, MA). Horse antimouse IgG was then applied (Vector Laboratories, Burlingame, CA), followed by an avidin-biotin-peroxidase complex (Vector Laboratories) and the chromagen 3′,3′-diaminobenzidine (with a counterstain of hematoxylin).

RESULTS

Transduction of SCC with Adenoviral Vector Containing a Reporter Gene (LacZ). We first determined the adenoviral vector concentration that provided maximum gene transduction without leading to nonspecific viral toxicity (Table 1). Using the LacZ reporter gene at a virus titer of 10 pfu, the transduction efficiency of SCCs, as evaluated by staining with β-galactosidase, varied from a low of 30% (SCC 9) to a high of 80% (Det562 and A431). At 50 pfu, efficiency increased to a range of 60–99%. There was little further increase in transduction at 100 pfu, with the exception of SCC 9, in which efficiency increased from 60% to 90%. We therefore performed our *in vitro* experiments using 50 pfu of virus.

Transduction with AS Cyclin D1. Cyclin D1 protein expression in the human SCC cell line A431 was decreased by 80% at 48 h after transduction with AS cyclin D1, as determined by a Western blot using a phosphorimager (Fig. 1*A*), whereas cells transduced with control vector LacZ, empty vector E1-treated cells, or mock-treated cells showed no decrease in cyclin D1 expression. The decreased expression of the mature cyclin D1 protein after treatment with AS cyclin D1 was paralleled by the increasing intensity of a M_r 21,000 band recognized by the cyclin D1 mAb (Fig. 1*A*).

Analysis of cyclin D1 protein expression in SCCs formed in SCID mice and injected with LacZ or AS cyclin D1 revealed a relatively uniform nuclear cyclin D1 protein expression in the LacZ-treated tumor (Fig. 1*B*), whereas in the AS cyclin D1-treated tumor, cyclin D1 expression was dramatically reduced (Fig. 1*C*).

Inhibition of Growth of SCC Cells after Transduction of AS Cyclin D1. The maximal effect of AS cyclin D1 in decreasing cell growth relative to LacZ or E1 infection was seen at 50 (A431, A253, SCC 13, Det562, and C33A) or 100 (SCC 9) pfu. Fig. 2 shows [^{3}H]thymidine incorporation after transduction of 50 pfu (or, for SCC 9, 50 and 100 pfu) of AS cyclin D1/cell relative to transduction with 50 pfu of LacZ/cell for each of the SCC cell lines. Compared to LacZ control, cell growth was suppressed in all but one (C33A) of the AS cyclin D1-treated cell lines, with suppression ranging from 37% for SCC 9 to 65% for Det562 (median suppression excluding C33A = 60%). C33A is a cervical SCC cell line with nonfunctional pRb (7). Transduction with E1 and

Table 1 *Transduction of human SCC with the LacZ reporter gene using an Ad (LacZ) for gene transfer*

For transduction of LacZ, SCC cells were infected with 10, 50, or 100 pfu/cell of LacZ. Seventy-two h later, cells were stained with β-galactosidase to determine the percentage of transduced cells.

	Percentage of cells transduced[a]		
SCC cell line	10 pfu	50 pfu	100 pfu
A431	80	99	99
A253	60	80	85
SCC 9	30	60	90
SCC 13	70	90	95
Det562	80	99	99
C33A	50	85	85

[a] The percentage of transduced cells is calculated from the total number of cells counted. In each case, a minimum of 100 cells in 10 randomly selected fields were counted.

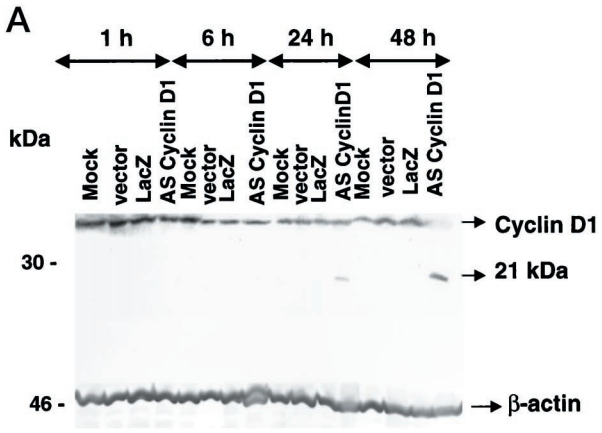
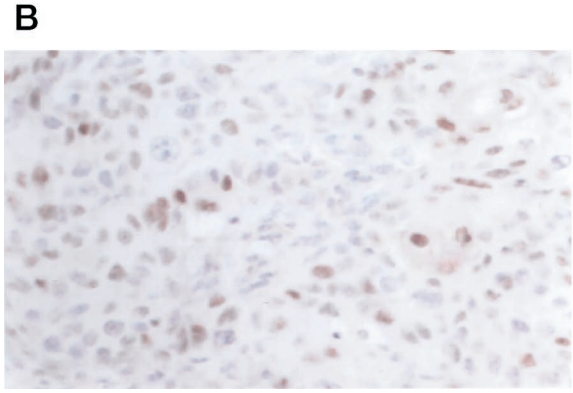
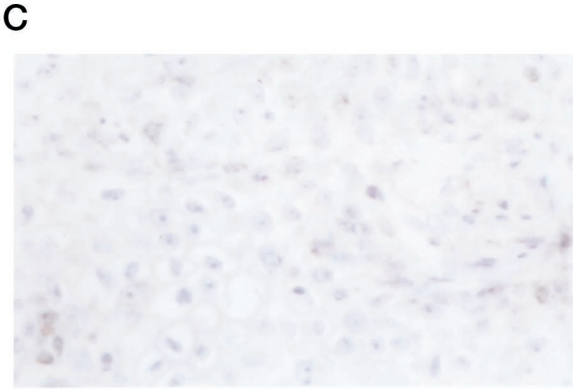


Fig. 1. Reduction of cyclin D1 protein expression in SCC cells after transduction of AS cyclin D1. *A*, Western analysis of cyclin D1 protein expression in SCC cells 1, 6, 24, and 48 h after transduction with either mock, vector only, LacZ, or AS cyclin D1, using an adenoviral vector at 50 pfu/cell. Total protein (100 μg) was probed with a cyclin D1 mAb. An 80% reduction of cyclin D1 was measured after 48 h in AS cyclin D1-treated cells when compared to LacZ controls. *21 kDa*, an M_r 21,000 product was recognized in AS cyclin D1-treated cells 48 h after transduction. *B*, cyclin D1 expression in an SCC grown in SCID mice and injected directly with LacZ at 5×10^8 pfu. Staining for cyclin D1 was performed after 48 h. Magnification, ×120. *C*, SCC injected with AS cyclin D1 and stained 48 h later. Magnification, ×120.





LacZ had minimal effect. AS cyclin D1 had a significant suppressive effect on each of the cell lines (SCC 9, SCC 13, and A253, $P = 0.01$; A431, $P = 0.002$; and Det562, $P = 0.001$), except for C33A. Whereas higher than optimal viral titers led to further growth suppression in the AS cyclin D1-treated cells in some instances, LacZ and/or E1 often had significant ($P < 0.05$) cell growth-suppressive effects.

With the MTT assay, the dose-response profile (data not shown) for A431, A253, SCC 9, and SCC 13 cells was similar to that seen in the [^{3}H]thymidine incorporation assay. At 50 pfu/cell, AS cyclin D1 exerted a significant suppressive effect on growth (A253, $P = 0.01$; A431 and SCC 9, $P = 0.008$; and SCC 13, $P = 0.004$).

In addition to our *in vitro* proliferation assays, we wished to evaluate the effect of AS cyclin D1 on preformed tumors. No toxicity was observed after intratumoral injection of either LacZ or AS cyclin D1. In each of the three cell lines tested, tumor growth was significantly decreased (SCC 13, $P = 0.004$; A253, $P = 0.005$; and A431, $P = 0.002$) after AS cyclin D1 treatment compared to tumors treated with LacZ. Fig. 3*A* illustrates the mean change in tumor volume over time, whereas Fig. 3*B* illustrates the change in tumor volume per day. Fig. 3*A* demonstrates that for A431, a fast-growing tumor, A253, a tumor with a moderate rate of growth, and SCC 13, a slow-growing tumor, AS cyclin D1 was equally effective not only in suppressing growth but also in leading to tumor shrinkage. In Fig. 3*B*, all tumors treated with AS cyclin D1 had a slower rate of growth than did the LacZ-treated tumors. Moreover, after treatment with AS cyclin D1, all tumors either shrank (15 of 16 tumors) or remained stable (1 of 16 tumors) in size, whereas after treatment with LacZ, 11 of 15 tumors grew in size, 2 of 15 tumors remained stable, and 2 of 15 tumors shrank minimally.

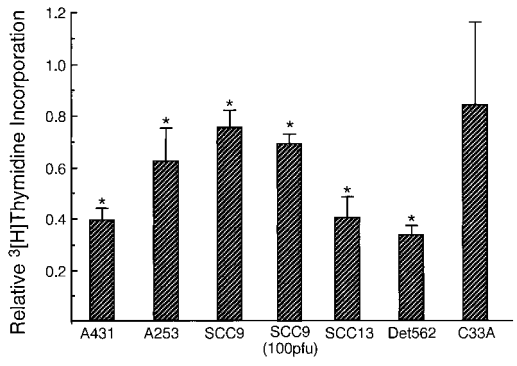


Fig. 2. *In vitro* inhibition of proliferation of SCC cells after transduction of AS cyclin D1. Relative [^{3}H]thymidine incorporation by SCC cells after transduction of AS cyclin D1 is shown. Results are shown for 50 pfu [optimal concentration for all cell lines except SCC 9, in which 100 pfu was the optimal concentration (both results are shown)]. No significant differences were found with mock-treated cells when compared to LacZ-transduced cells. Transduction with 100 pfu was not significantly different from that with 50 pfu-treated cells, but some cell lines demonstrated nonspecific growth suppression. ∗, the result is significantly different than LacZ control ($P = 0.01$–0.001).

Apoptosis Is Induced after Transduction of AS Cyclin D1. Cell cycle arrest in G_1 was not seen after treatment with AS cyclin D1. Even if G_1 arrest had been present, this would not adequately explain the shrinkage of preformed tumors. In order for tumors to shrink, cell death must occur. We therefore hypothesized that the cells were undergoing apoptosis due to treatment with AS cyclin D1. Twenty-four h after transduction with AS cyclin D1, the percentage of sub-G_0 (apoptotic) SCC 13, A253, and Det562 cells was significantly greater than in the mock-treated and LacZ-treated groups, and this percentage remained higher at 48 and 72 h (Fig. 4*A*). For A431 and SCC 9 cells, a significantly greater fraction of apoptotic cells was first noted 48 h after AS cyclin D1 infection ($P = 0.008$–0.001). A significant increase in the percentage of apoptotic C33A cells, which lack functional pRb, was not detected after treatment with AS cyclin D1.

In s.c. tumors formed in SCID mice, staining indicative of apoptosis

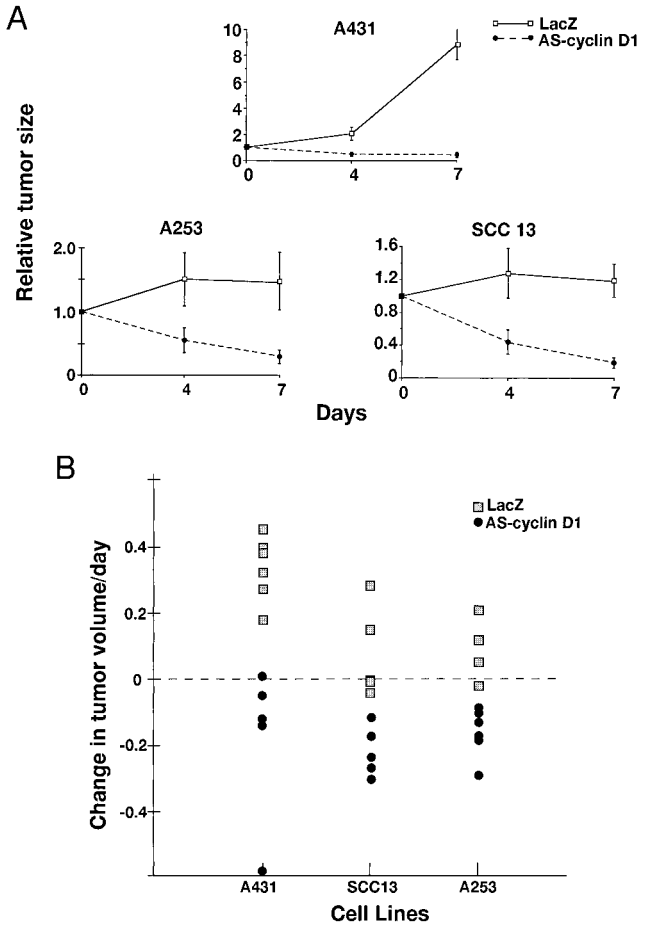


Fig. 3. Inhibition of SCC growth after *in vivo* transduction of AS cyclin D1. Tumors were grown to a volume of 100 mm^3 and then injected once with 50 μl of 5×10^8 pfu of adenoviral vectors. *A*, mean change in tumor size after injection of LacZ or AS cyclin D1 over a 7-day period. *B*, estimated change in size per day over a 7-day period for each tumor after a single transduction of AS cyclin D1 or LacZ. Each point represents the result of one animal.

was rare in the LacZ-treated tumor cells (Fig. 4*B*, *i*), whereas most of the tumor cells treated with AS cyclin D1 stained positively (Fig. 4*B*, *ii*).

DISCUSSION

Our hypothesis was that cyclin D1 was critical to squamous cell carcinogenesis, and that suppression of cyclin D1 protein expression would lead to growth arrest. To test the hypothesis, we created an adenoviral vector containing cyclin D1 in an AS orientation, speculating that the AS product would suppress cyclin D1 protein production in the SCC cells. After treatment with AS cyclin D1, cyclin D1 protein production was substantially suppressed both *in vitro* and *in vivo*. With the reduction in the M_r 34,000 cyclin D1 protein, a M_r 21,000 band recognized by the mAb to cyclin D1 appeared. To determine whether the M_r 21,000 band was more likely to be a functional truncated protein or a breakdown product, we again mock-infected or infected A431 cells with LacZ or AS cyclin D1 and waited 48 h before harvesting. With overnight exposure to substrate, a M_r 21,000 band was observed in each of the three lanes (data not shown). From this we surmised that the smaller band detected in both control and treated cells is more likely a breakdown product of cyclin D1 rather than a functional truncated protein. Others (30) have demonstrated an *in vitro* reduction in cyclin D1 mRNA by 24 h and in protein by 48 h after treatment of ovarian cancer cells with AS oligonucleotides to cyclin D1. Similarly, we have demonstrated an 80% reduction 48 h after infection. The reduction in cyclin D1 expression after treatment with AS cyclin D1 is coincident with a significant increase in apoptosis.

We also evaluated the effect of AS cyclin D1 on cell growth and apoptosis in six SCCs. In all cell lines with functional pRb, cell growth was significantly decreased, both *in vitro* and *in vivo*. In all cell lines with functional pRb, a significantly greater fraction of cells had undergone apoptosis 48 h after the addition of AS cyclin D1 than after treatment with LacZ. Compared to the control, a nonsignificant increase in apoptosis was seen in a cell line lacking functional pRb 72 h after treatment with AS cyclin D1, consistent with a report documenting the effect of another G_1 regulator, $p16^{INK4A}$, on cells with and without functional pRb (31).

In all SCCs tested, there was not only a reduction in tumor size compared to control, but tumor shrinkage relative to baseline as well. This response after *in vivo* treatment of preformed tumors is very promising, because prior reports of *in vivo* treatment of SCCs using wild-type p53 (32, 33), rather than demonstrating tumor shrinkage, document a delay in tumor growth after a single injection or stable disease after repeat therapy. We are aware of the nonspecific toxicity that occurs after

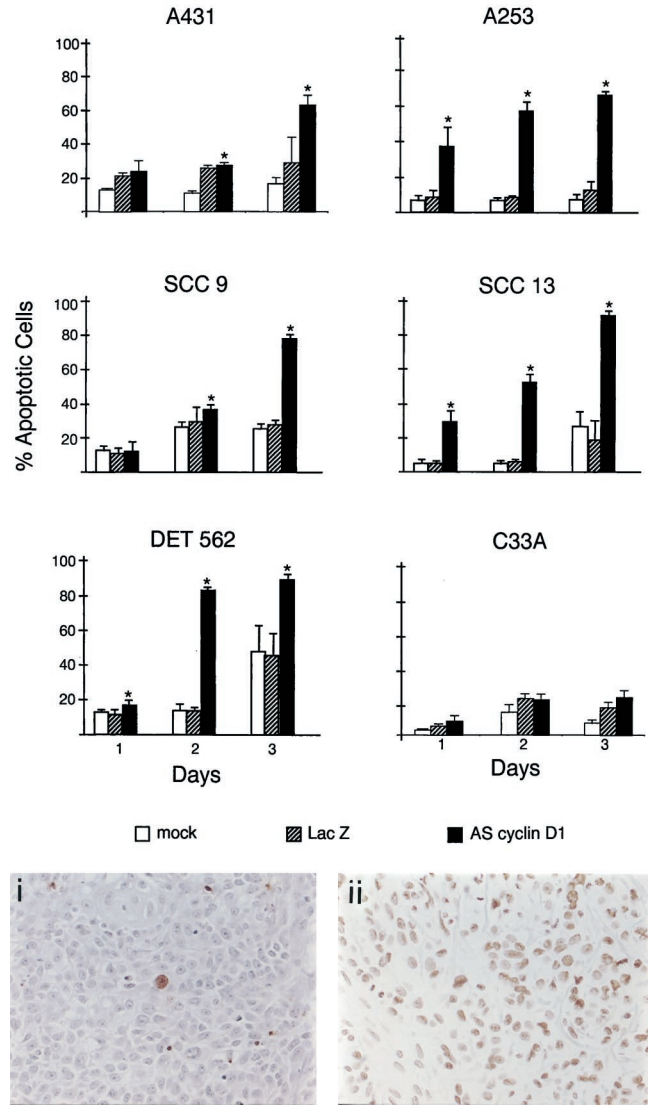


Fig. 4. Induction of apoptosis in SCC cells *in vitro* and *in vivo* after transduction of AS cyclin D1. *A*, percentage of apoptotic cells *in vitro* 1, 2, and 3 days after mock, LacZ, or AS cyclin D1 transduction at 50 pfu. For each cell line, the fraction of apoptotic cells after treatment with AS cyclin D1 was significantly greater ($P = 0.008-0.001$) than after mock or control virus treatment. *, the result is significantly different from the LacZ control. *B*, apoptosis in tumors formed in SCID mice after the injection of 10^7 A253 cells and subsequent treatment with LacZ (*i*) or AS cyclin D1 (*ii*). Magnification, ×120.

infection with E1- or E1/E3-deleted adenoviral vectors regardless of the cDNA expression cassette, including G_2 cell cycle arrest (34). This is why, in addition to a mock control, we had both an E1-deleted virus without an expression cassette and an E1/E3-deleted virus containing β-galactosidase cDNA as controls. It is not surprising, therefore, that a decrease in [^{3}H]thymidine incorporation, in absorbance with the MTT assay, and in tumor growth was noted after infection with the E1- and/or E1/E3-deleted adenoviral control at higher pfu titers. AS cyclin D1 induced significant growth suppression and increased apoptosis over and above the effects of the adenoviral controls.

Prior reports have demonstrated *in vitro* (30, 35–38) and/or *ex vivo* (39–41) inhibition of cyclin D1 protein expression and decreased cell growth with AS cyclin D1. A variety of transfection techniques were used, including calcium phosphate coprecipitation, lipofection, and retroviral infection. Our plan was to evaluate the ability of AS cyclin D1 to inhibit cell growth in a model system that we felt to be most clinically relevant: that of preformed tumors derived from human cancer cells. We chose a human tumor system in which the importance of cyclin D1 has been clearly demonstrated, SCCs, in which the gene is amplified in up to half of the tumors, and RNA and protein are overexpressed in most cases. Our findings document for the first time, to our knowledge, that *in vivo* transduction with AS cyclin D1 leads to tumor reduction, and that this effect is due, at least in part, to an increase in apoptosis.

We chose an Ad as the transduction vehicle because infection is very reliable, as documented by LacZ staining of 80–100% of cells infected at a pfu of 50–100 for each of the SCC cell lines studied. Calcium phosphate and lipofection techniques are not as reliable in infecting human cells (42). Moreover, unlike retroviral vectors, the adenovirus does not have the theoretical risk of inducing cancer and is effective whether or not the cell is dividing. Protein production from a CMV-driven promoter reliably yields high protein production for maximal effect.

Throughout this report, we have chosen A431 and A253 as the prototype SCCs, and we have augmented our findings by analyzing additional SCCs. The response of SCC cells to AS cyclin D1, as evaluated by cell growth, appears to be related to the infectability of the cells (Table 1), their level of cyclin D1 protein expression, and their pRb status, given that a major cause of cell transformation involves the disruption of pRb-mediated constraints on G_1 progression (8, 43). pRb is functional in the vast majority of both primary (44) and immortalized SCCs (45, 46), including all cell lines used in the present study except C33A. AS cyclin D1 treatment had little or no effect on C33A. Of the cell lines that did respond to AS cyclin D1, SCC 9 and A253 demonstrated the least growth reduction by [^{3}H]thymidine incorporation. These two cell lines, by β-galactosidase staining after rejection with LacZ, appear to have less viral entry into the cells compared to the other cell lines. The cell line with the greatest response, Det562, showed intense staining in virtually 100% of cells and has a high expression of cyclin D1 protein (data not shown). Thus, the already encouraging response of SCCs to cyclin D1 AS treatment might be further enhanced by designing a viral construct that permits increased entry into cells. A recent report (47) suggests that cells with limited adenoviral infectability might be made more receptive to viral entry by the addition of the CAR (coxsackie and adenovirus receptor) gene. We also considered the potential influence of baseline expression of other cell cycle regulators, including p16 and cyclin E, and the presence or absence of cyclin D1 amplification on response to treatment with AS cyclin D1. Based on the limited results we obtained for p16 (48), cyclin D1 amplification, and cyclin E (data not shown), none of the three appeared to influence the response of SCCs to AS cyclin D1.

Before our *in vivo* studies, we presumed that AS cyclin D1 treatment would lead to cell cycle arrest, resulting in delayed tumor growth. When we noted tumor shrinkage, we began to search the literature for evidence that cyclin D1 induced apoptosis. Two recent reports that had not been published when we performed our experiments suggest that cyclin D1 may have a role in cell survival, although they have conflicting findings. Both studies measured cyclin D1 protein expression in the rat brain; the first study measured cyclin D1 protein expression after injury (49), and the second study (50) measured cyclin D1 protein expression after occlusion of blood flow and resulting brain ischemia. In the first study, cyclin D1 was not seen in the apoptotic cells, whereas in the second study, cyclin D1 expression was increased in the early stages of apoptosis. Our results support these findings that cyclin D1 protein is involved in cell survival and suggest that a decrease in expression may lead to cell death.

There is substantial evidence suggesting that the genes that trigger transformation may also be involved in programmed cell death. Altered expression of c-Myc, E2Fs, and adenovirus E1A may cause cell transformation but can also cause apoptosis (51). Moreover, overexpression of oncogenes or proto-oncogenes may lead to the transformation of proliferation-competent cells (51) and to apoptosis in quiescent or terminally differentiated cells. The overexpression of cyclin D1 through the transduction of a CMV-driven expression vector containing cyclin D1 leads to apoptosis in fibroblasts, renal cells, and neuronal cells (51). Moreover, the accelerated entry into S phase associated with up-regulation of cyclin D1 after the injection of granulocyte colony-stimulating factor leads to the induction of apoptosis in C2M myeloid leukemia (52). This dual role of oncogenes and proto-oncogenes initially appears confusing. However, it has been observed (51) that many tumor cell lines with very high mRNA levels of cyclin D1 only moderately overexpress the protein. Thus, the absolute amount of cyclin D1 present within the cell may determine the cellular response to the protein, with moderately high levels leading to increased growth, and extremely high levels leading to apoptotic cell death.

Other potential mechanisms through which AS cyclin D1 might work are the concepts of gene addiction and gene hypersensitivity (53). It has been observed that inhibiting cyclin D1 expression in either esophageal or colon cancer cells markedly inhibits growth, despite the fact that the treated cells continue to express levels of cyclin D1 protein that are greater than those in tumor cells of similar origin lacking cyclin D1 overexpression at baseline. Thus, tumors with cyclin D1 overexpression may be addicted to the protein, requiring high levels for survival. The concept of gene hypersensitivity relates to the suppressive effect of adding a tumor suppressor gene to a cancer cell lacking the gene. Although other gene alterations exist, restoration of the single suppressor gene is often sufficient to revert a malignant phenotype toward a normal phenotype (54), suggesting that the cells are hypersensitive to the function of the missing gene.

Our findings document the following: (*a*) treatment with AS cyclin D1 leads to a decrease in cyclin D1 protein production over time; (*b*) an optimal dose of AS cyclin D1 with minimal viral toxicity can be identified; (*c*) AS cyclin D1 treatment leads to apoptosis; and (*d*) AS cyclin D1 can suppress the growth of a wide range of human SCCs, including those from the face, head and neck, and vulva. Our data indicate that SCC growth suppression is mediated through direct suppression of cyclin D1 expression, leading to the shrinkage of tumors.

Injecting formed tumors more closely recreates the clinical situation than experiments documenting tumor shrinkage after *ex vivo* therapy. SCCs of the skin (both facial and vulvar) and of the head and neck can be visualized and remain local in the vast majority of cases. As such, these tumor types are readily accessible to direct injection with AS cyclin D1. AS cyclin D1 treatment may prove to be an appropriate adjunct to currently available therapy, especially when standard therapy has not proven effective or no additional treatment (such as ionizing radiation) can be tolerated. Indeed, even if AS treatment does not cause a tumor to vanish, significant shrinkage would still complement the efficiency of standard treatment such as surgery and/or radiation.

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